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PRINCIPAL INVESTIGATOR: Edith M. Lord, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14627

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Edith M. Lord, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester
Rochester, New York 14627

email - **Edith_Lord@urmc.rochester.edu**

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13. ABSTRACT *(Maximum 200 Words)*
PSA, which is used as a diagnostic marker for prostate cancer, is also expressed at low levels by female breast tissue. PSA secretion by human breast tumors has been found to be associated with an improved prognosis. In addition, a correlation has been found between breast tumors with a high number and density of microvessels and metatastic disease. This information along with the demonstration that PSA can prevent endothelial cell proliferation and migration led us to hypothesize that PSA might be serving as an anti-angiogenic factor. Determining whether mouse or human tumors transfected to express PSA grew at a different rate than parental tumors tested this. The tumors were mouse tumors expressing PSA were found to grow at a slightly slower rate. The tumors were also examined for the number and density of blood vessels by staining tissue sections for the endothelial cell marker, CD31. Although the morphology of the vessels in the PSA expressing tumors differed from the parental tumors, the tumors did not appear to be markedly more hypoxic. It is possible that the PSA must be activated before it can affect angiogenesis within the growing tumors.

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INTRODUCTION

Breast cancer is the second most prevalent cancer for women in this country with over 192,000 cases and 39,000 deaths last year. Although breast cancer death rates have declined in recent years due primarily to improved screening and treatment, it is clear that better treatment strategies are needed to combat this disease. Knowing more about the formation and growth of these tumors is necessary in order to rationally design better treatments. Breast carcinomas, like other tumors, require the formation of new blood vessels for growth past a few millimeters in size [1]. This process, known as angiogenesis, appears to be stimulated by the progressive growth of the malignant cells which outgrow the capacity of the existing vasculature to provide needed oxygen and other nutrients. The low oxygen or hypoxic conditions that result from the consumption of oxygen by the growing cells triggers a cascade of events that up regulate pro-angiogenic factors and down regulate anti-angiogenic factors such that new vessels are formed to support the continued growth of the tumor. Interestingly, studies have shown that breast tumors with a high density of microvessels tend to be more metastatic than tumors with fewer vessels and thus have a worse prognosis [2, 3]. Results such as these have reinforced the idea that therapies that could limit or restrict the formation of blood vessels within tumors might have a marked effect on not only primary tumors, but also on distant metastases. For these reasons, considerable effort has been devoted to identifying and testing new anti-angiogenic agents. The results of these studies have been somewhat controversial, but efforts continue and currently 80 of these agents are in clinical trials. The effectiveness of these therapies remains to be seen, but it would appear that some of these have been moved to large clinical trials too quickly and would have benefited from more extensive preclinical studies. Clearly the mechanism of angiogenesis is complex, and the factors affecting it are not fully understood. The purpose of this research was to determine if prostate specific antigen (PSA), a protease produced mainly by prostate, but also present in some breast cancers, could affect angiogenesis and growth of breast cancer cells.

BODY

We became interested in reports indicating that prostate specific antigen (PSA), a protease found in the prostate, might have an anti-angiogenic effect. Although expressed primarily by the prostate in males, PSA is also expressed in female breast tissue at low levels [4]. In addition, it was reported that breast tumors expressing PSA were associated with an improved prognosis [3]. For these reasons we decided to determine if endogenous production of PSA by tumors cells could 1) alter tumor growth rate of breast carcinomas or 2) alter the vascularization within growing tumors. To approach these questions, we selected two breast carcinoma cell lines, one of BALB/c mouse origin (EMT6) and one of human origin (MCF-7). Each of these cells lines was transfected with the gene for human PSA and clones stably expressing the antigen were selected and PSA expression verified using an ELISA assay. PSA expressing EMT6 clones were easily obtained and a clone that produces 45 ng/ml PSA when 2 x 10^5 cells are plated in 2 ml of media for 48 hrs was selected for further study (EMT6/PSA.1). PSA expressing MCF-7 have been more difficult to produce, perhaps due to their much slower growth

rate, and we have been unable, as yet, to produce a PSA expressing MCF-7 line. For this reason, we are now using another human breast carcinoma cell line, T-47D. This line has been transfected and is currently being cloned. Because of this difficulty with the human cell lines, most of the data presented in this report has been obtained using the mouse cell line.

Parental EMT6 and EMT6/PSA.1 cells (2 x 10^5) were implanted intramuscularly (i.m.) into the rear leg of syngeneic BALB/c mice and tumor growth monitored. The EMT6/PSA.1 tumors grew with kinetics very similar to that of the parental cell line (Figure 1). These cell lines have been implanted in a similar fashion into athymic nude mice, which lack functional T cells and are thus unable to mount an immune response to the tumors. In the immunologically intact BALB/c mice it is possible that an immune response by the mice to the human PSA antigen could affect the growth of the tumors. Thus, it is worthwhile to determine the growth rate under conditions in which immunity is much less of a factor. This experiment is still in progress. Nevertheless, at this point we would conclude that PSA has little or no effect on tumor growth, even in BALB/c mice, thus making it a suitable model for study.

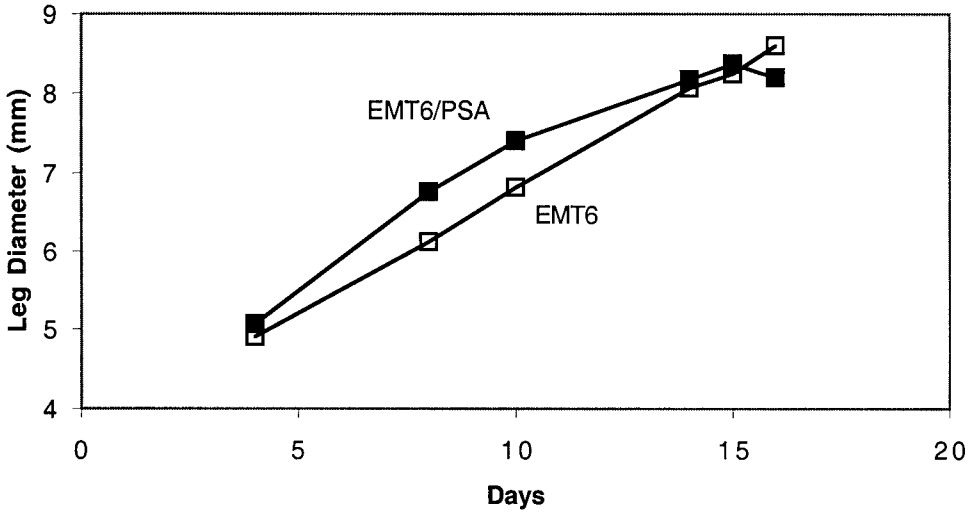

Figure 1. EMT6 and EMT6/PSA have similar growth rates in syngeneic BALB/c mice. Tumor cells (2 x 10^5) were injected i.m. in the rear flank of the mice and tumor growth monitored by measuring the leg diameter over time. Each line represents average tumor growth in a group of three mice.

We initiated experiments to directly address the question of possible differences in the vascularization of the parental compared to the PSA expressing tumors. We had initially planned to use standard immunocytochemistry on frozen sections of tumors to assess blood vessels and the oxygenation status of the tumors, techniques with which we have considerable experience. However, this approach has many limitations in that sectioning the frozen tumors is a laborious and time-consuming task, and the antibodies do not always work well on fixed tissue. Thus, to improve this procedure, we modified a whole mount technique that is easier to perform and provides a more complete view of

the vessel morphology. This is whole mount microscopy technology allows visualization and characterization of blood vessels and other cell types in small pieces of viable unfixed tissue. Because it was necessary to validate this new approach, the initial experiments were performed with the B16 cell line, a melanoma of C57BL/6 origin. This cell line was chosen because it is syngeneic with a special transgenic mouse that expresses the green fluorescent protein (GFP) under the control of the MHC class I promotor. This mouse is very useful in that the cells, which are of host origin, such as the vascular endothelial cells that compose the blood vessels are GFP positive, whereas the implanted tumor cells are GFP negative. It is thus relatively easy to see the blood vessels without the need to stain the tissue. Interesting results were obtained in these preliminary experiments in that although there was considerable heterogeneity within the tumor tissue as is often seen, the B16/PSA expressing tumors appeared to have fewer vessels than the parental B16 tumors. Figure 2 shows representative photographs of these.

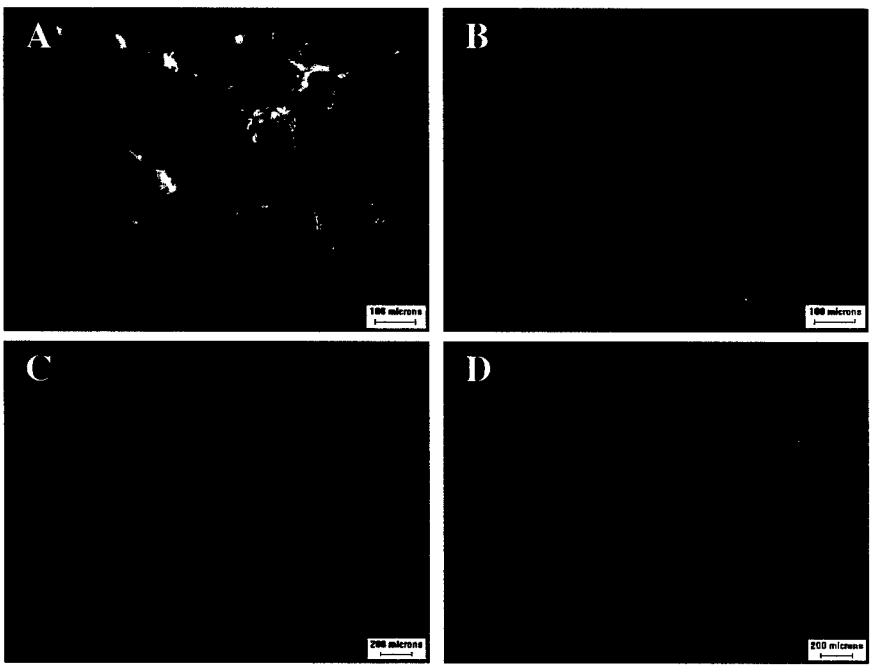

Figure 2: B16 and B16/PSA tumors exhibit different vascular patterns. B16 (A,C) or B16/PSA (B,D) tumor cells (2 x 10^5) were injected i.m., the tumors allowed to grow for 14 days and then removed. Small pieces of the tumors were imaged for GFP expression (A, B) or stained with phycoerythrin (PE)-conjugated anti-CD31 antibody and then imaged.

The other value of this model was that it allowed us to also validate the use of antibodies to stain structures within the tumors. We have determined that the antibodies can penetrate these small tissue pieces and effectively stain the blood vessels as shown in Figure 2 for the anti-CD31 staining of the blood vessels. Indeed, using the antibodies allowed even clearer visualization than did the endogenous GFP.

After verifying that the antibodies could be used effectively to image structures within the tumors, we then analyzed the breast tumors expressing PSA or not. These studies with the EMT6 and EMT6/PSA tumors also revealed interesting differences in the

vasculature. The appearance of the vasculature in the EMT6 tumors is distinct from that of the B16 tumors in that the vessels are extremely contorted, and many areas of the tumors are almost devoid of vessels. In contrast, many of the vessels within the EMT6/PSA tumors are more like those in normal tissue (Figure 3).

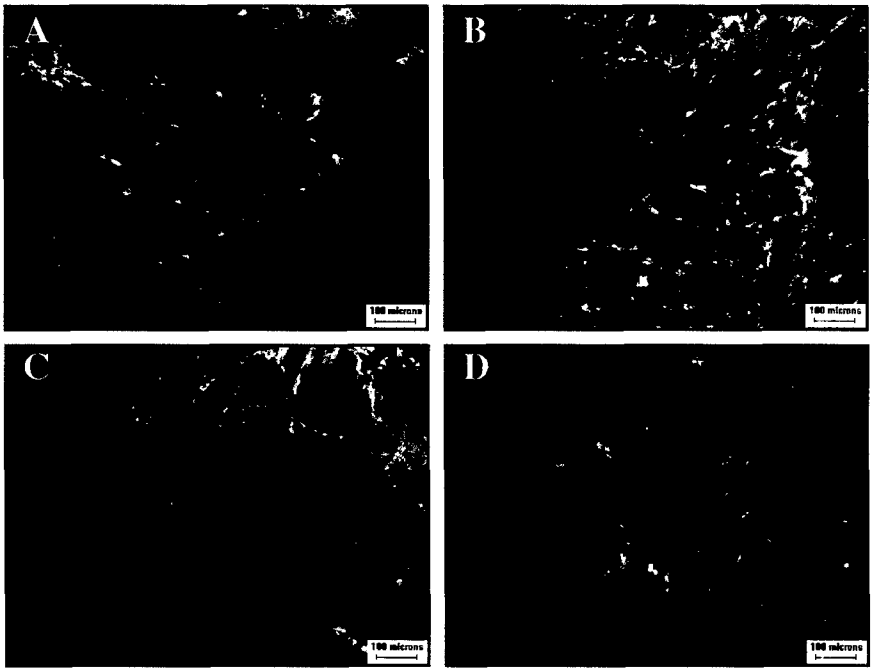

Figure 3: Vascular patterns in EMT6 and EMT6/PSA tumors. EMT6 (A,B) or EMT6/PSA (C,D) tumor cells (2×10^5) were injected i.m., the tumors allowed to grow for 14 days and then removed. Small pieces of the tumors were stained with phycoerythrin (PE)-conjugated anti-CD31 antibody and then imaged.

An exciting possibility is that angiogenesis has not occurred in these tumors, but rather the tumors are co-opting the existing normal vessels and using those to obtain nutrients. This is reminiscent of a recent report in a rat glioma model [5]. In this system tumors were observed to initially be very well vascularized due to their ability to use existing host vessels, but these vessels later regressed resulting in massive tumor cell death, which was subsequently rescued by angiogenesis at the tumor margin. This appeared to result from the high expression of angiopoietin-2, which destabilizes the vessels, followed by expression of vascular endothelial cell growth factor (VEGF), which supports the formation of new vessels. In light of this model, it is interesting that some regions of the EMT6/PSA tumors, appear to be in a transition from the normal appearing vessels to vessels more similar to those in the EMT6 tumors (Figure 3D). It will be extremely interesting to determine if intratumoral expression of PSA affects the relative expression levels of these two important growth factors. Although beyond the scope of the original grant, these experiments are currently being performed.

The effects of PSA that we have observed on tumor growth in vivo appear to be somewhat modest. However, it is difficult to precisely compare our results to the earlier reports, which were limited largely to studies on endothelial cells grown in vitro and to an indirect measure of tumor growth in vivo [6]. Obviously, the marked differences in the systems used to assess effects of PSA could account for differences in the level of the effects observe. It is also possible that enzymatic activity of the PSA could be involved. Although the information regarding the functional activities of PSA in the context of angiogenesis are somewhat limited, it is known to be a serine protease with chymotrypsin-like activity [7]. It is one member of the kallikrein family of serine proteases. It has been shown to cleave and inactivate insulin-like growth factor binding protein-3 (IGFB-3), which could increase the availability of highly mitogenic insulin-like growth factors (IGFs) and thus stimulate tumor growth [8]. However, PSA itself is secreted in a zymogen form that must be cleaved prior to have activity. This can be accomplished by a second member of the kallikrein family, hK2 [9], as well a perhaps by other proteases. In the prostate both hK2 and PSA are present, and thus the PSA may be primarily present as an active enzyme. However, in the PSA expressing breast carcinoma lines we have produced, only PSA is present and thus it may be only partially active. If enzymatic activity is required for PSA to act as an anti-angiogenic factor, then it would have been diminished in our experiments. Although not part of the original grant, to determine if this is the case, we plan to transfect tumor cells lines with both PSA and hK2 and examine the vascular patterns when these cells are grown as tumors in syngeneic mice. This also raises the general question of how protease affect the growth of blood vessels. Given the technique of gene transfer we have used and the whole mount technique we have developed, it will be possible to further pursue these important questions.

KEY RESEARCH ACCOMPLISHMENTS

1. Development of whole mount technique for visualization of blood vessels in tumors.
1. Validation of the use of antibody staining to detect and characterize structures within growing tumors using the whole mount technique.
1. Production of PSA expressing mouse and human cell lines.
1. Detection of differences in vascularization between parental and PSA expressing tumors.

REPORTABLE OUTCOMES

1. Scott Gerber obtained a Master of Science degree from the University of Rochester Department of Microbiology & Immunology based in part on work supported by this grant.
1. Production of PSA expressing mouse (EMT6) and human (MCF-7) cell lines.
1. Funding from the United States Public Health Service was applied for to further develop the whole mount technology developed and funded by the current grant.

CONCLUSIONS

1. Expression of PSA by breast carcinomas does not significantly alter the growth rate of breast carcinomas.
2. Expression of PSA by breast carcinomas does appear to change the blood vessel formation patterns within the tumors, however, how this affects function remains to be determined.
3. The possibility that PSA must be activated by another enzyme for optimal anti-angiogenic activity may explain the failure to see more extensive changes in the growth of PSA expressing tumors.

Bibliography

Gerber, S., Simon, D., Frelinger, J.G. and Lord, E.M. Alteration of breast tumor vasculature by endogenously expressed prostate specific antigen. (in preparation).

Personnel

No personnel received pay from this research effort.

REFERENCES

1. Folkman, J., *Angiogenesis in cancer, vascular, rheumatoid and other disease.* Nat Med, 1995. **1**(1): p. 27-31.
2. Weidner, N., et al., *Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma.* New England Journal of Medicine, 1991. **324**(1): p. 1-8.
3. Yu, H., et al., *Prognostic value of prostate-specific antigen for women with breast cancer: a large United States cohort study.* Clin Cancer Res, 1998. **4**(6): p. 1489-97.
4. Diamandis, E.P., H. Yu, and D.J. Sutherland, *Detection of prostate-specific antigen immunoreactivity in breast tumors.* Breast Cancer Res Treat, 1994. **32**(3): p. 301-10.
5. Holash, J., et al., *Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF.* Science, 1999. **284**(5422): p. 1994-8.
6. Fortier, A.H., et al., *Antiangiogenic activity of prostate-specific antigen.* J Natl Cancer Inst, 1999. **91**(19): p. 1635-40.
7. Ban, Y., et al., *The proteolytic activity of human prostate-specific antigen.* Biochem Biophys Res Commun, 1984. **123**(2): p. 482-8.
8. Cohen, P., et al., *Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma.* J Clin Endocrinol Metab, 1992. **75**(4): p. 1046-53.
9. Kumar, A., et al., *Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2.* Cancer Res, 1997. **57**(15): p. 3111-4.

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